Stable vesicles made from new triple-chain amphiphiles: long-term stability toward leakage of the trapped substances

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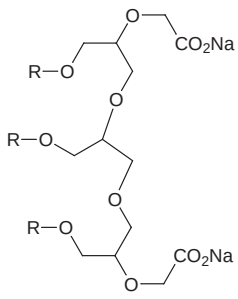
Very stable vesicles, which can keep 5(6)-carboxyfluorescein trapped inside for months, are obtained from triple-chain amphiphiles bearing two carboxylate groups derived from 1-*O*-alkylglycerols.

Numerous endeavours to construct stable liposomes or vesicles have been made. There are two classical approaches to suppressing the leakage of trapped substances: one is the addition of other molecules, such as cholesterol[1] or cholesterol polysaccharides,[2] to bilayer systems, and the other is the introduction of special group(s) or structures into the hydrophobic moiety of the amphiphiles. As examples of the latter, unique amphiphiles bearing polymerisable functional groups[3] or phytanyl moieties[4] in the hydrophobic chains have been reported. It should be noted that most of these specially designed amphiphiles are based on the conventional 'double-chain' structure, like phospholipids.

A series of novel amphiphiles bearing three hydrophobic alkyl chains and two hydrophilic head groups has been designed and prepared previously by the authors' group.[5] It was found that these 'triple-chain' compounds showed a greater ability to lower surface tension and to form micelles at lower concentrations than the corresponding 'double-chain' surfactants bearing two ionic head groups. These results suggest that the three alkyl chains in the molecule can make a positive contribution to the surface-active properties because both the inter- and intramolecular hydrophobic interactions are strengthened compared to those of the corresponding double-chain surfactants.[6] This speculation prompted us to prepare vesicles made from the triple-chain amphiphiles and to compare their ability to suppress the leakage of substances trapped inside with that of vesicles made from conventional phosphatidylcholines.



1 R = *n*-$C_{16}H_{33}$

2 R = *n*-$C_{18}H_{37}$

The triple-chain amphiphiles used in this work (**1** and **2**) were bis(carboxylate)s prepared from 1-*O*-alkylglycerols.[5]† Dipalmitoyl- and distearoyl-phosphatidylcholines (DPPC and DSPC, respectively; Nippon Fine Chemical Co., 99.8%) were also used as the reference lipids because these phosphatidylcholines (PCs) bearing saturated acyl chains are known to form relatively stable bilayer membranes at room temperature.[7] Small unilamellar vesicles containing concentrated 5(6)-carboxyfluorescein (CF) in an aqueous buffer solution were prepared by the conventional hydration–sonication method.‡ Fig. 1 shows the released percentage of CF from vesicles made from phospholipids or triple-chain amphiphiles during storage at 40 °C.

Upon comparing the results for the triple-chain amphiphiles with those for the corresponding phospholipids bearing the same number of carbon atoms in one hydrophobic chain (**1** *vs.* DPPC, **2** *vs.* DSPC, respectively), we found that the vesicles made from the triple-chain amphiphiles were much more stable toward the leakage of trapped CF. In particular, vesicles made from **2** released less than 10% of the CF after 12 months under the experimental conditions used in this work. The transition temperatures from the gel state to the liquid crystal state (T_c), measured *via* DSC, of aqueous dispersions of these lipids or amphiphiles are as follows: 45.3 °C (for **1**), 59.3 °C (**2**), 41.1 °C (DPPC) and 56.3 °C (DSPC). In the case of vesicles made from **1** or DPPC, the relatively fast release of trapped CF may be attributed to the high fluidity of the membrane because the T_c of these two compounds is only a little higher than the storage temperature. The large difference in the ability to suppress the leakage between **2** and DSPC, however, cannot be explained by the T_c value.

The microfluidity of the bilayer membrane was estimated by the established method using pyrene as a fluorescent probe.[8] The I_e/I_m ratio (I_e and I_m are fluorescence intensities of the pyrene eximer at 468 nm and the pyrene monomer at 394 nm, respectively) increases as the microfluidity of the hydrophobic phase in the membrane increases. The relation between the I_e/I_m ratio and the measured temperature is shown in Fig. 2. [conditions: amphiphile or lipid (10 mM), pyrene (0.15 mM), 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), excitation at 335 nm]

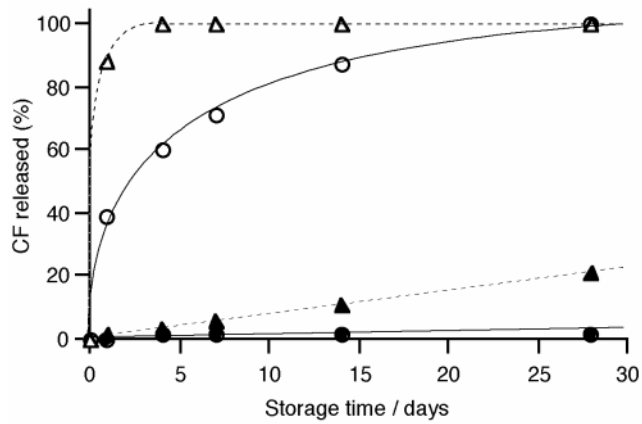


Fig. 1 Release (%) of 5(6)-carboxyfluorescein (CF) trapped inside vesicles of (△) DPPC, (▲) DSPC, (○) **1** and (●) **2** as a function of storage time (days) at 40 °C.

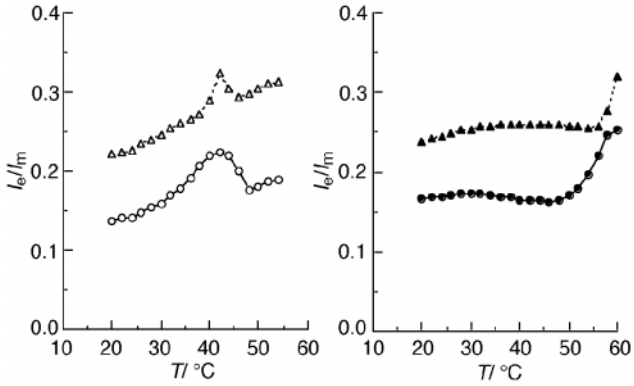


Fig. 2 Effect of temperature on the I_e/I_m ratio of pyrene buried in the membrane of (△) DPPC, (▲) DSPC, (○) **1** and (●) **2** vesicles. Conditions: amphiphile or lipid (10 mM), pyrene (0.15 mM), 100 mM NaCl, 20 mM Tris-HCl (pH 7.5), excitation at 335 nm.

Although the I_e/I_m ratios for **1** and DPPC increased gradually from 30 °C up to about 45 °C, there was very little change in the ratios for **2** and DSPC until 50 °C. These results agree well with the T_c of each compound. It is noteworthy that the I_e/I_m ratio of the triple-chain amphiphile is much lower than that of the corresponding phospholipids bearing the same number of carbons in a hydrophobic chain, meaning lower microfluidity of the membrane made from the triple-chain amphiphiles than that of the phospholipids. In summary, it is possible to say that the long-term stability of vesicles made from **2** toward the leakage of trapped CF may result from the three much more closely packed octadecyl chains in the bilayer of **2**, as compared to other lipids.

Because the triple-chain compounds in this work have two carboxylate groups, vesicles made from these amphiphiles are expected to have some pH-sensitive functions. Detailed investigation of their vesicles under various pH conditions is now in progress.

Notes and references

† The structure and purity of new compounds **1** and **2** were confirmed using the corresponding dimethyl esters because bis(carboxylate) compounds **1** and **2** were hygroscopic. *Selected data* for the dimethyl ester of **1** (R = *n*-$C_{16}H_{33}$), mp 78–79.5 °C (from EtOH); δ_H(400 MHz; $CDCl_3$) 0.88 (t, 9 H), 1.25–1.77 (m, 84 H), 3.40–3.60 (m, 21 H), 3.75 (s, 6 H), 4.35 (m, 4 H); *m/z* (FAB) 1095 [(M+K)$^+$, 100%], 1057 [(M+1)$^+$, 4] (Calc. for $C_{63}H_{124}O_{11}$: C, 71.54; H, 11.81. Found: C, 71.52; H, 11.92%). For the corresponding dimethyl ester of **2** (R = *n*-$C_{18}H_{37}$), mp 80–81 °C (from EtOH); δ_H(400 MHz; $CDCl_3$) 0.88 (t, 9 H), 1.17–1.60 (m, 96 H), 3.34–3.62 (m, 21 H), 3.73 (s, 6 H), 4.33 (m, 4 H); *m/z* (FAB) 1095 [(M+K)$^+$, 100%], 1057 [(M+1)$^+$, 4] (Calc. for $C_{69}H_{136}O_{11}\cdot H_2O$: C, 71.45; H, 11.99. Found: C, 71.55; H, 11.95%).

‡ A film of lipid or amphiphile (40 μmol) was prepared on the inside wall of a test tube by evaporation of its $CHCl_3$ solution and stored in a desiccator overnight under reduced pressure. After addition of 4 ml of a Tris-HCl buffer (20 mM, pH 7.5) containing 100 mM of CF to the test tube, the mixture was vortex-mixed for 10 min and successively sonicated for 5 min at about 10 °C higher than its T_c using a probe-type sonicator under a stream of nitrogen. Small unilamellar vesicles containing trapped CF were separated from untrapped CF by eluting the vesicle dispersion through a Sephadex G-50 gel column with 20 mM Tris-HCl buffer containing 100 mM of NaCl (pH 7.5). The formation of vesicles from compounds **1** and **2** was confirmed by a well-established gel-filtration method (ref. 9). Thus two fractions containing CF were observed in a series of eluates. The first fraction, which was eluted with an excluded volume of the column, indicated the presence of particles including a water phase separated from the outside phase. The second fraction contained a large quantity of untrapped CF only. This was also the case for DPPC and DSPC experiments in this work. The amount of CF released (%) from the vesicles was calculated by means eqn. (1),

$$\text{CF released } \% = (I_x - I_0)/(I_t - I_0) \times 100 \quad (1)$$

where I_0 is the fluorescence intensity of the vesicle suspension containing CF at initial time, I_x is the intensity of the suspension after a definite period of storage, and I_t is the fluorescence intensity after addition of an aqueous solution of Triton X-100 (100 g l^{-1}) to the suspension. The fluorescence intensity at 530 nm was measured at 25 °C using an excitation wavelength at 490 nm.

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